Developmental Biology 376 (2013) 171–186

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Loss of *ascl1a* prevents secretory cell differentiation within the zebrafish intestinal epithelium resulting in a loss of distal intestinal motility

Gillian Roach [a], Rachel Heath Wallace [a], Amy Cameron [a], Rifat Emrah Ozel [b], Cintia F. Hongay [a], Reshica Baral [a], Silvana Andreescu [b], Kenneth N. Wallace [a,*]

[a] Department of Biology, Clarkson University, 8 Clarkson Ave., Potsdam, NY 13699, USA
[b] Department of Chemistry, Clarkson University, 8 Clarkson Ave., Potsdam, NY 13699, USA

ARTICLE INFO

Article history:
Received 4 July 2012
Received in revised form
5 January 2013
Accepted 8 January 2013
Available online 23 January 2013

Keywords:
Intestine
ascl1a
Enteroendocrine
Enterocyte
Enterochromaffin
Goblet cell
Serotonin
Notch
Motility
Spatotemporal mapping
Smooth muscle
Enteric neurons
Differential pulse voltammetry

ABSTRACT

The vertebrate intestinal epithelium is renewed continuously from stem cells at the base of the crypt in mammals or base of the fold in fish over the life of the organism. As stem cells divide, newly formed epithelial cells make an initial choice between a secretory or enterocyte fate. This choice has previously been demonstrated to involve Notch signaling as well as Atonal and Her transcription factors in both embryogenesis and adults. Here, we demonstrate that in contrast to the *atoh1* in mammals, *ascl1a* is responsible for formation of secretory cells in zebrafish. *ascl1a*−/− embryos lack all intestinal epithelial secretory cells and instead differentiate into enterocytes. *ascl1a*−/− embryos also fail to induce intestinal epithelial expression of *deltaD* suggesting that *ascl1a* plays a role in initiation of Notch signaling. Inhibition of Notch signaling increases the number of *ascl1a* and *deltaD* expressing intestinal epithelial cells as well as the number of developing secretory cells during two specific time periods: between 30 and 34 hpf and again between 64 and 74 hpf. Loss of enteroendocrine products results in loss of anterograde motility in *ascl1a*−/− embryos. 5HT produced by enterochromaffin cells is critical in motility and secretion within the intestine. We find that addition of exogenous 5HT to *ascl1a*−/− embryos at near physiological levels (measured by differential pulse voltammetry) induce anterograde motility at similar levels to wild type velocity, distance, and frequency. Removal or doubling the concentration of 5HT in WT embryos does not significantly affect anterograde motility, suggesting that the loss of additional enteroendocrine products in *ascl1a*−/− embryos also contributes to intestinal motility. Thus, zebrafish intestinal epithelial cells appear to have a common secretory progenitor from which all subtypes form. Loss of enteroendocrine cells reveals the critical need for enteroendocrine products in maintenance of normal intestinal motility.

Introduction

The intestinal epithelium is a unique vertebrate tissue that continually renews itself throughout the life of the organism. Epithelial cells originate from stem cells at the base of the crypt in mammals (Spence et al., 2011) or the base of the fold in zebrafish (Ng et al., 2005; Wallace et al., 2005b) and differentiate into enterocytes, goblet, enteroendocrine, or paneth cells as they move out of the stem cell compartment. As cells reach the tip, they undergo apoptosis and are shed into the lumen. Due to the constant turnover of epithelial cells, typical neural innervation is not common; instead, axons terminate near the epithelial cells (Gershon, 2003). In order for luminal conditions to be communicated to enteric neurons and surrounding tissue, a variety of specialized secretory cells differentiate within the epithelium and release their products to the basal surface (Gunawardene et al., 2011; Moran et al., 2008). These secretions alter motility and intestinal absorption.

During mammalian intestinal epithelial development, cells in the developing proliferative compartments at the base of the villi express Atoh1/Math1. Loss of *atoh1/math1* prevents differentiation of secretory cells (Yang et al., 2001). In contrast, Hes1 is expressed along the villi, is excluded from secretory cells, and loss of *hes1* results in increased secretory cell differentiation (Jensen et al., 2000). Notch signaling components are expressed in the embryonic intestine and loss of either *atoh1/math1* or *hes1* results in misregulation of Delta ligands (Jensen et al., 2000; Yang et al., 2001). This suggests a model of lateral inhibition in which epithelial cells expressing *atoh1/math1* differentiate into secretory cells. Differentiating secretory cells then express Notch ligands to induce the enterocyte fate in surrounding cells by activating the Notch signaling pathway. Activation of the Notch receptor results

* Corresponding author. Fax: +1 315 268 7118.
E-mail address: kwallace@clarkson.edu (K.N. Wallace).

0012-1606/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.01.013

in cleavage of the intracellular domain (ICD), which enters the nucleus to interact with RBP-Jk resulting in the activation of downstream genes such as *hes1*, which in turn represses genes specifying the enteroendocrine fate (Fre et al., 2011; Hartenstein et al., 2010; Schonhoff et al., 2004).

This mechanism of choice between secretory cells and enterocytes has been conserved over a wide range of species. Loss of Notch signaling in zebrafish fails to specify enterocytes and many more secretory cells develop (Crosnier et al., 2005). In the adult Drosophila intestine, Notch signaling is required to produce enterocytes, while low Notch levels produce secretory cells (Bardin et al., 2010; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007).

Notch continues to play an important role in the adult intestine where signaling is required for both maintenance of the stem cell compartment and making the choice between the secretory or enterocyte fate. Recent work demonstrates that stem cells expressing a Leucine-rich repeat containing G protein–coupled receptor 5 (Lgr5) cluster around Paneth cells (Sato et al., 2011). Paneth cells are required for proliferation and maintenance of the stem cell compartment and are a source of Wnt3, Notch ligand Dll4, EGF, and TGF-A (Sato et al., 2011). Loss of Notch signaling results in absence of crypt proliferation and differentiation of stem cells into goblet cells (Van Es et al., 2005). In addition, as during embryogenesis, *atoh1/math1* is required for epithelial cells to enter the secretory fate (Van Es et al., 2010). Notch signaling is induced in cells entering the enterocyte fate with up regulation of *hes*1 and repression of *atoh*1/*math*1.

After initial differentiation into enterocyte and secretory lineages, there is further restriction into specific subsets of each cell type. Neurogenin3 specifies the enteroendocrine lineage (Jenny et al., 2002), while other secretory cells differentiate into goblet or Paneth cells. Goblet cells produce mucin to lubricate the lumen and provide a barrier to microbial invasion. Enteroendocrine stem cells go on to differentiate into approximately 10 different lineages (Schonhoff et al., 2004). Subtypes of enteroendocrine cells are dispersed throughout the epithelium (Sjolund et al., 1983) and use G protein coupled receptors, similar to oral taste receptors, to sense luminal contents, resulting in the stimulation of mediator release (Iwatsuki and Uneyama, 2012; Raybould, 2010). Mediators from enteroendocrine cells act on mucosal enteric neurons and surrounding tissue to alter secretory and motor activity within the intestine (Hansen and Witte, 2008; Mawe et al., 2006; Spiller, 2011). While enterocytes develop specific regionalization along the proximal to distal axis, specific classes of these cells are not as well defined.

One well-studied enteroendocrine lineage is enterochromaffin (EC) cells, which synthesize and secrete serotonin (5HT). EC cells act as transducers, releasing 5HT in response to stretch and pressure (Heredia et al., 2009) as well as luminal contents (Kidd et al., 2008), resulting in altered motility and stimulation of mucosal secretions. In mammals, fourteen 5HT receptors have been identified: 5HT1A, 5HT1B, 5HT1P, 5HT2A, 5HT2B, 5HT3, and 5HT4 are expressed within the digestive system (Hansen and Witte, 2008). 5HT receptors are located on both intrinsic and extrinsic neurons as well as surrounding tissue including smooth muscle (Hansen, 2003; Hansen and Witte, 2008; Mawe et al., 2006). Binding to 5HT1P, 5HT3, and 5HT4 is excitatory while binding to 5HT1A is inhibitory (Gershon and Tack, 2007; Hansen and Witte, 2008).

Here we investigate the role of one of the zebrafish acheate-scute like family members, *ascl1a,* in specification of the intestinal epithelial secretory lineage. *ascl1a* expression suggests a role in specification of secretory cells. We find that *ascl1a* null mutants do not develop secretory cells and the entire epithelium differentiates into enterocytes. *ascl1* has been shown to play a role in avian, mammalian, and zebrafish *delta* expression (Hans and Campos-Ortega, 2002; Mizuguchi et al., 2006; Nelson et al., 2009). We find that *ascl1a* mutants fail to initiate *deltaD* expression within the intestinal epithelium, suggesting that *ascl1a* expressing cells activate Notch signaling in neighboring cells. Here we investigate whether Notch signaling is active throughout the entire period of intestinal *ascl1a* expression. In addition, *ascl1a* mutants have a loss of anterograde motility. Replacement of 5HT initiates motility with the same velocity, distance, and frequency found in wild type embryos. Pharmacological removal of 5HT in wild type embryos does not, however, result in loss of anterograde motility.

Materials and methods

Fish Stocks

Fish maintenance and matings were performed as previously described (Westerfield, 1993). AB wild type fish were used for most procedures (Westerfield, 1993). *ascl1a* null mutants were obtained from Matthias Hammerschmidt and described in (Pogoda et al., 2006). Embryos kept for motility experiments were treated with pronase (Roche) at the end of the first day of embryogenesis and washed out at the beginning of the second day of embyrogenesis in order to remove the chorion. Embryos were allowed to grow with pigment and E3 (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) was exchanged each day for optimal growth.

Immunohistochemistry

Embryos were fixed in 4% formaldehyde for a period of either 2 hours or overnight. Fixed embryos were then permeabilized with Proteinase K (Sigma) or Collagenase (Sigma) in PBS (0.(186.5 mM NaCl; 2.68×10^{-2} mM KCl; 1 mM Na_2HPO_4 (dibasic); 6.95 mM NaH_2PO_4-H_2O (monobasic)) for 20 min at room temperature. Primary antibody was added and incubated at 4 °C overnight. Embryos were then washed and incubated with secondary antibody (1:500, Molecular Probes-Invitrogen) for two hours. Embryos for desmin and acetylated tubulin antibody incubation were permeabilized as previously described with phospholipase A (Sigma) (Akhtar et al., 2009). Primary antibodies are rabbit serotonin (5HT) (1:500, Sigma), mouse 2F11 (1:1000, AbCam), rabbit anti-type IIb sodium-phosphate cotransporter (1:100 dilution, a gift of A. Werner), rabbit anti-desmin (1:100 dilution, Sigma), mouse anti-acetylated tubulin (1:100 dilution, Sigma), mouse anti-HuC/D (1:50 dilution, Molecular Probes), mouse anti-ZO1 (1:100 dilution, gift of Dr. S. Tsukita and T. Obara), rabbit anti-sodium/potassium ATPase (1:100 dilution, Developmental Studies Hybridoma Bank).

For Wheat Germ Agglutinin experiments (1:100, Vector Laboratories) embryos were permeabilized with Collagenase and incubated overnight in PBST (186.5 mM NaCl; 2.68×10^{-2} mM KCl; 1 mM Na_2HPO_4 (dibasic); 6.95 mM NaH_2PO_4-H_2O (monobasic); 0.1% Tween-20). Embryos were visualized on a Nikon TE200 inverted microscope using a Hamamatsu Orca camera with IP lab software.

At least three independent experiments were performed for all immunohistochemical detections.

Embryo visualization

The digestive system was visualized as described previously (Olden et al., 2008). For immunohistochemistry, intestines were dissected and mounted separately in Vectashield (Vector Laboratories). For RNA *in situ* hybridization, skin and yolk were removed and visualized ventrally in glycerol. Both intestine dissection and